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Coliphage MS2 containing 5-Fluorouracil

II. RNA-deficient Particles formed in the Presence of 5-Fluorouracil

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The addition of 5-fluorouracil to *Escherichia coli* infected with MS2 leads to the formation of two classes of fluorouracil-containing phage: one with buoyant density greater than that of normal MS2 and another with buoyant density lower than normal. The latter are non-infectious and do not adsorb to *E. coli* receptor sites as measured by inhibition of infective center formation. Serologic tests revealed slight differences between normal and defective phage, but no difference in the fingerprints of the coat proteins was detectable. The RNA content of defective phage is about 65% of normal. The isolated RNA is heterogeneous, in keeping with the density heterogeneity of the particles, and has an average S_{20} value of 20. Although the RNA is not infectious, it is more active than normal MS2 RNA in directing the synthesis of phage-specific proteins in cell extracts. As with normal MS2 RNA, the major product is phage coat protein, which yields a normal fingerprint. The results suggest that the RNA fragment lacks a specific part of the phage RNA molecule.

1. Introduction

We have reported that addition of 5-fluorouracil to a culture of *Escherichia coli* previously infected with the RNA bacteriophage MS2 results in the production of particles with a buoyant density lower than normal (Shimura, Moses & Nathans, 1965). Since this effect of FU† might shed light both on its mode of action in altering the expression of messenger RNA (Champe & Benzer, 1962) and on the development of MS2, we have studied the defective particles in more detail. Our results indicate that these particles are deficient in RNA and are adsorbed poorly by host cells. In these respects, the FU-induced particles closely resemble the defective particles produced by certain amber mutants of related RNA phages (Lodish, Horiuchi & Zinder, 1965; Heisenberg, 1966; Argetsinger & Gussin, 1966). Although the RNA fragment isolated from the FU-induced particles is not infectious, it serves as an active template for the synthesis of phage-specific proteins in cell extracts.

2. Materials and Methods

MS2 and its host, *E. coli* C3000, were obtained from R. L. Sinsheimer. The procedures for growth, purification and assay of phage were those described previously (Shimura *et al.*, 1965). An arginine-lysine double auxotroph of *E. coli* C3000 was obtained by the penicillin method (Gorini & Kaufman, 1960) after ultraviolet irradiation. This strain is designated C3000-25.

† Abbreviations used: FU, 5-fluorouracil; p.f.u., plaque-forming units; T_m, melting temperature.

Protein synthesis in *E. coli* extracts and analysis of the products were as described previously (Nathans, 1965), except that electrophoresis of tryptic peptides was done at pH 3·6 in pyridine acetate (pyridine–acetic acid–water, 1 : 10 : 280).

Determination of protein was by the phenol method (Lowry, Rosebrough, Farr & Randall, 1951) using bovine serum ablumin as a standard. Total RNA was determined by the orcinol method (Schneider, 1957) and base composition, by optical density of the 2′, 3′-nucleotides following hydrolysis of the RNA with KOH and separation of the nucleotides by paper electrophoresis at pH 3·5 (Markham & Smith, 1952).

Source of chemicals. 5-Fluorouracil was generously supplied by Dr R. Duschinsky of Hoffman–LaRoche, Inc., Nutley, N.J. Uniformly labeled [^{14}C]amino acids were obtained from the New England Nuclear Corp,. Boston, Mass., and tritiated amino acids were from the Nuclear Chicago Corp., Chicago, Ill. or from Schwarz BioResearch, Inc., Orangeburg, N.Y.

3. Results

(a) *Formation of defective particles*

We have reported previously that, in addition to heavy phage particles, particles of low buoyant density in CsCl are formed when FU is added to cultures of *E. coli* previously infected with MS2 (Shimura *et al.*, 1965), as seen in Fig. 1. (A similar

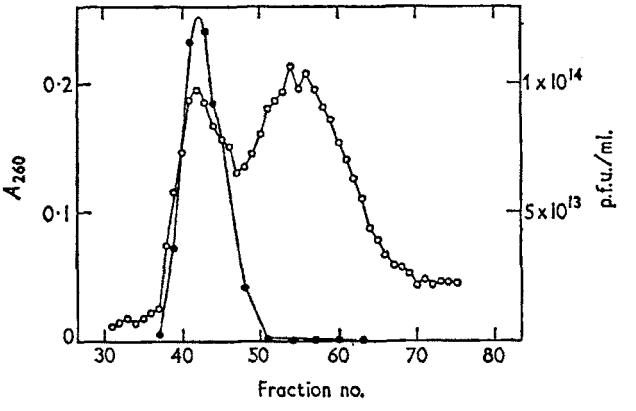

FIG. 1. CsCl gradient equilibrium sedimentation of FU particles.

E. coli C3000 was infected with MS2 as previously described (Shimura *et al.*, 1965) and 30 μg FU/ml. added 17 min after infection. The progeny phage were purified as described (Shimura *et al.*, 1965) and the CsCl fractions analysed for infective phage (●) and A_{260} (○). Normal MS2 gave a single optical density peak with slight tailing on the light side.

observation was reported with f2 by Lodish *et al.*, 1965.) At the concentration of FU used in these experiments, 80% of the uracil is replaced by FU (Shimura *et al.*, 1965). These light particles are not infectious (Fig. 1), and in keeping with their low buoyant density, they are deficient in RNA, the ratio of RNA to protein being 0·19, compared with 0·29 for normal MS2 prepared in the same way. By electron microscopy, the normal and defective particles are indistinguishable (kindly carried out by Dr E. Moudrianakis). If the protein content of light particles is assumed to be normal, the average RNA content is about two-thirds of normal. The breadth of the light particle peak (Fig. 1) indicates that there is considerable variation in RNA content of different particles, however, and re-banding of particles from the heavier, middle and lighter regions of the peak yields populations of distinct densities. Also, as noted below, sedimentation of the RNA shows multiple components.

(b) *Properties of the protein of the defective particles*

Since the low RNA content of the light particles might be secondary to abnormal, poorly protective coat protein resulting in degradation of the phage RNA by nucleases present in the lysate, the coat protein was analyzed in various ways. First it was shown that the coat protein was synthesized predominantly, if not entirely, after the addition of FU (Fig. 2). In this experiment, radioactive amino acids were added either

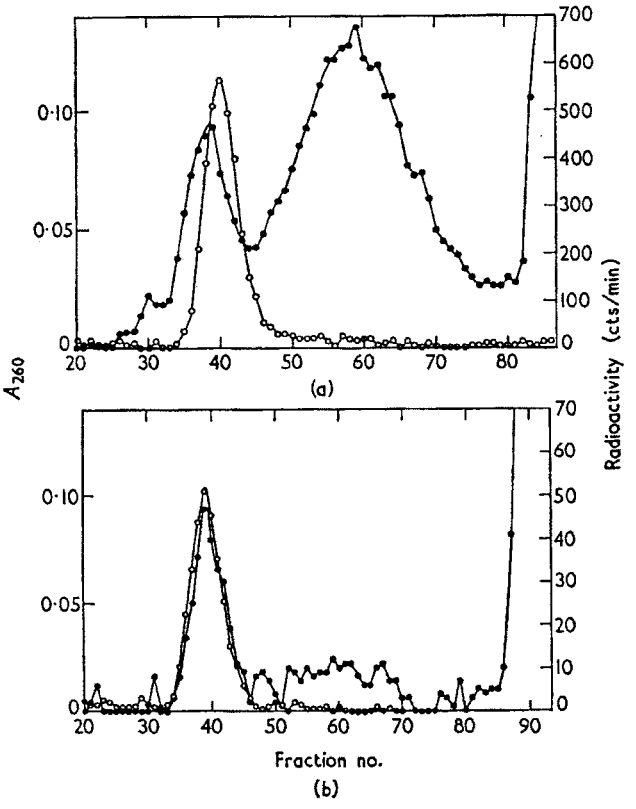

FIG. 2. Incorporation of [^{14}C]amino acids into defective particles.

E. coli C3000-25, growing in medium containing lysine and arginine, was infected with MS2 at an input multiplicity of 10. 12 min after infection, the cells were centrifuged and (a) suspended in unsupplemented medium or (b) in medium containing [^{14}C]lysine and [^{14}C]arginine. At 16 min excess [^{12}C]lysine and arginine were added to (b) and at 18 min, 30 μg FU/ml. was added to both (a) and (b). At 22 min [^{14}C]lysine and [^{14}C]arginine were added to (a) and each culture was lysed at 60 min. After addition of carrier MS2, the phage were isolated as described in Shimura *et al.*, 1965. The Figures represent analyses of CsCl gradients; ●, [^{14}C]protein, ○, A_{260} of carrier phage.

before FU (and quenched shortly before FU addition), or after FU. As shown in Fig. 2, radioactivity appears in light particles only when labeled amino acid is added after FU.

Fingerprint analysis of the tryptic peptides of the protein of defective particles labeled with [^{14}C]lysine and [^{14}C]arginine was carried out as previously described (Nathans, 1965). When the [^{14}C]peptides were compared with normal peptides stained with ninhydrin and labeled with tritiated lysine and arginine, the peptides

from defective particles were normal in mobility and in relative amount. By this rather insensitive technique, therefore, the coat proteins of normal and defective particles were indistinguishable.

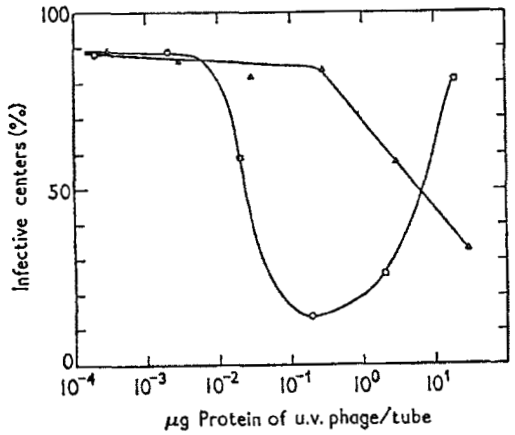

FIG. 3. Inhibition of infective-center formation by ultraviolet-inactivated MS2 and FU particles.

Varying amounts of inactivated phage particles were mixed with 5×10^7 bacteria at 37°C and 1 min later 5×10^5 p.f.u. of normal MS2 added. After 5 min, antiserum against MS2 was added and 5 min later infective centers assayed. The Figure shows the number of infective centers relative to the control value with inactivated phage: ○, with irradiated MS2; △, with irradiated FU particles. The inactivated phage had < 1 p.f.u. per 20 μg protein.

Another test of the properties of the protein of light particles was an indirect test of adsorption to bacteria—the ability to inhibit infection of *E. coli* by normal MS2 (Lodish *et al.*, 1965). As shown in Fig. 3, there is a striking difference between normal and defective particles in this test. In each case the phage particles were treated with sufficient ultraviolet to inactivate normal phage to < 1 p.f.u. per 0·265 A_{260} unit. In the case of inactivated MS2, 50% inhibition occurs with a phage concentration equiva-

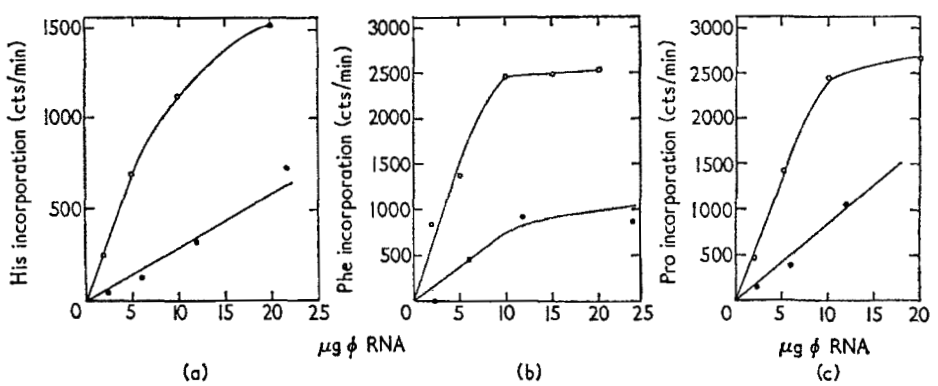

FIG. 4. *In vitro* incorporation into protein of [^{14}C]histidine (a). [^{14}C]phenylalanine (b) and [^{14}C]proline (c) stimulated by RNA from defective particles (○) and from intact MS2 (●). Incubations were carried out as previously described (Nathans, 1965).

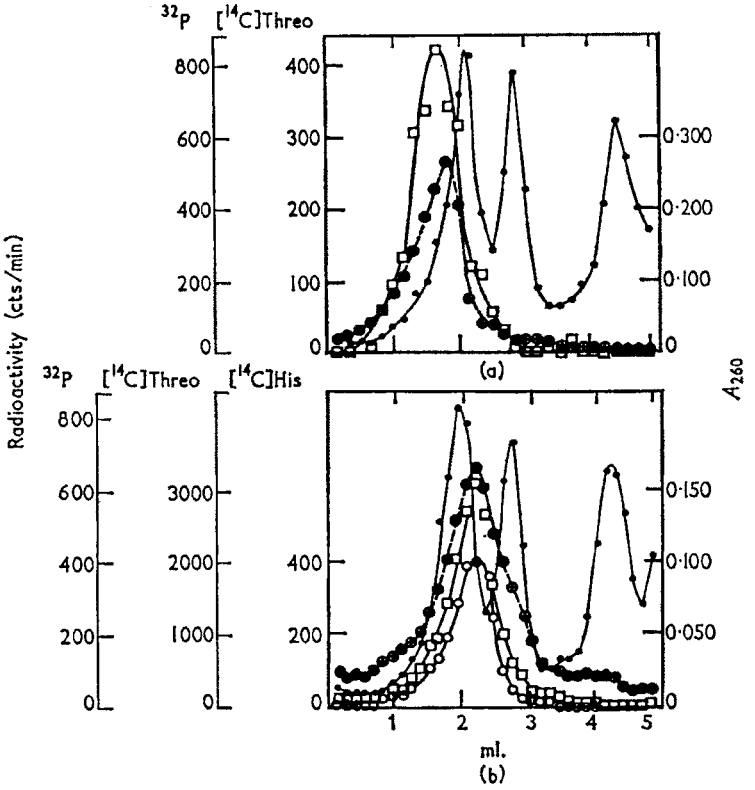

FIG. 5. Sedimentation and messenger activity of RNA from (a) normal MS2 and (b) from defective particles.

Samples of phage RNA were mixed with ^{32}P-labeled *E. coli* RNA and centrifuged through a 5 to 20% sucrose gradient at 35,000 rev./min for 4·5 hr in a Spinco SW39 rotor. Each fraction was tested for stimulation of incorporation of [^{14}C]threonine (□) and [^{14}C]histidine (○). A_{260} (⊕) and ^{32}P marker RNA (●) are also given. The [^{14}C]histidine incorporation directed by normal RNA is not plotted, but corresponded with the [^{14}C]threonine peak.

lent to about 0·025 μg protein, whereas about 6 μg of light particle protein is required. This result indicates that light particles adsorb poorly to bacterial receptor sites. The secondary rise in infective centers with normal phage is presumably due to binding of antibody used in the infective center assay, thus allowing unadsorbed testing phage to yield infective centers. In contrast, equivalent amounts of defective particles do not neutralize all the added antibody, indicating a difference in serum-binding activity between normal and defective particles. A difference in serum binding capacity was also detected by measuring the neutralizing activity of antiserum after a five-minute incubation with ultraviolet-inactivated MS2 or defective particles; more defective phage was required to bind a given amount of antiserum in this test.

(c) *Properties of the RNA of defective particles*

As already noted, the RNA isolated from defective particles is heterogeneous; the bulk of the RNA had an S_{20} value of 20 in 0·01 M-Tris HCl (pH 7·4) and 0·01 M-magnesium acetate, compared with a value of 27 for MS2 RNA. The base composition was not different from normal MS2 RNA (except for fluorouracil), and the melting curve was essentially identical with that of intact MS2 RNA containing FU (Shimura *et al.*, 1965), i.e. the T_m was 61·5°C and there was about 10% less total hyperchromicity compared with normal MS2 RNA.

Although the RNA was not infectious for spheroplasts (method of Davis, Pfeifer & Sinsheimer, 1964, modified by Strauss, 1964), it was more active than normal MS2 RNA by a factor of two to three in stimulating protein synthesis in *E. coli* extracts (Fig. 4). As shown in Fig. 4, incorporation into protein of histidine, an amino acid not found in MS2 coat protein, is stimulated to about the same extent as that of amino acids present in coat protein (proline and phenylalanine). That the stimulation is due to the fragments of RNA from defective particles is shown in Fig. 5, which compares the sedimentation in sucrose and activity of RNA from normal MS2 (Fig. 5(a)) and from defective particles (Fig. 5(b)). As seen in the Figure, the activity of the RNA fragments for incorporation of both histidine and threonine

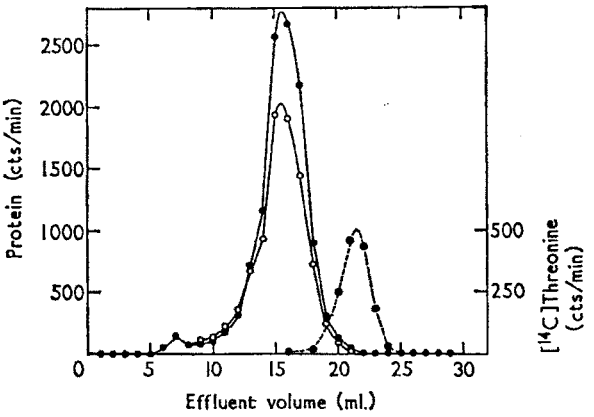

FIG. 6. Sephadex chromatography of threonine-labeled protein, synthesized *in vitro*.

Chromatography was carried out as previously described (Nathans, 1965). ●, [^{3}H]Threonine-labeled protein made with normal MS2 RNA; ○, [^{14}C]threonine-labeled protein made with FU-particle RNA; ⊕, free [^{14}C]threonine marker. The dominant peak corresponds to phage coat protein.

(present in phage coat) corresponds to the optical density peak. Also evident in the Figure is the enhanced activity of the RNA fragments; the difference in activity is exaggerated, however, because of the inhibitory effect of high concentrations of sucrose.

The protein products made under the direction of the fragments of phage RNA were analysed by electrophoresis and chromatography of the tryptic peptides and by fractionation on Sephadex G200 (Nathans, 1965). The fingerprint of the tryptic peptides showed all the soluble coat protein peptides in their usual position and in the same relative amounts as the peptides of the protein made with normal MS2 RNA. As shown in Fig. 6, on Sephadex G200 the coat protein peak constituted about 80% of the total protein, which is similar to the result obtained when normal RNA was used as a template.

4. Discussion

(a) *Production of defective particles*

The mechanism of formation of defective FU particles is best explained by the observations of Lodish *et al.*, (1965), Heisenberg (1966) and Argetsinger & Gussin (1966) that a certain class of mutants of the RNA coliphage gives rise in the non-permissive host to RNA-deficient particles. In fact, Lodish *et al.* independently observed that FU mimicked this mutation. Our results with FU particles resemble their findings with sus-1 defective particles in that both show poor adsorption to host cells and are indistinguishable from normal phage by electron microscopy. In the case of FU particles, although a fingerprint of the coat protein failed to reveal any difference from normal MS2 protein, there was a difference in serum-blocking power of the intact particles. The suggestion that defective mutant particles are formed because of non-functional "RNA-protecting" protein (or "maturation" protein) would also explain the formation of defective virus particles in the case of 5-fluorouracil. An alternate possibility is that the FU particle has abnormal coat protein subunits which do not fit as compactly as normal coat, thereby allowing nucleases to degrade the RNA (see Hummeler & Wecker, 1964). Since FU probably gives rise to a large variety of changes in all proteins made on progeny RNA in the infected cell, it is possible that there are minor changes in the amino acid sequence of the coat protein of the FU-particles detectable serologically, but because of their inconstant occurrence, not detectable by the chemical analyses we applied.

The failure of FU particles to adsorb to *E. coli* receptor sites may be due to abnormalities in coat protein or, more likely, to a defect in the protein required for viral "maturation" (Lodish *et al.*, 1965; Heisenberg, 1966; Argetsinger & Gussin, 1966). The normal phage appears to have a small quantity of the latter protein (Nathans, Oeschger, Eggen & Shimura, 1966), which may serve as the site of attachment of the phage to the bacterial cell.

(b) *RNA fragment as a messenger*

The high activity of the RNA of defective particles as a messenger in directing the synthesis of phage-specific proteins in cell extracts suggests that this RNA consists of a rather specific piece of the molecule, including possibly the 5′ or initiating end of the molecule. This argument is strengthened by the recent finding that a particular phage protein (probably the RNA synthetase) is not detectable when the FU-RNA fragment is the template (Shimura, unpublished results). The simplest

interpretation of this observation is that the cistron for this protein is located at one end of the phage RNA, which has been cleaved from the rest of the molceule in the formation of the RNA-deficient particle.

(c) *Effect of 5-fluorouracil in messenger RNA*

In the FU-RNA used as template for the synthesis of proteins, about 80% of the uracil was replaced by FU (Shimura *et al.*, 1965). In spite of this high degree of replacement, the synthetic phage-specific proteins fractionated normally on Sephadex, and fingerprint analysis of the synthesized coat protein showed a peptide map indistinguishable from that of the coat protein made with normal MS2 RNA as template. These observations are in agreement with those of Grunberg-Manago & Michelson (1964), who failed to find errors of translation with FU-containing synthetic polynucleotides. Our methods for detecting amino acid changes are rather insensitive, however, and would require errors greater than 10% for a given peptide. These results, therefore, do not exclude the possibility that errors in translation result from the presence of FU in messenger RNA. More critical information is likely to result from studies of the expression of FU-containing MS2 RNA in infected cells.

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